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Is Bone Equally Responsive to Calcium and Vitamin D Intake from Food vs. Supplements? Use of 41Calcium Tracer Kinetic Model

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Use of ^{41}Calcium Tracer Kinetic Model

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List of abbreviations used: AI, adequate intake; AMS, accelerator mass spectrometry; ANOVA, analysis of variance; BAP, bone specific alkaline phosphatase; BMC, bone mineral content; BMD, bone mineral density; BMI, body mass index, ^{41}Ca, calcium-41; CTx, serum C terminal telopeptide of type 1 collagen; CV, coefficient of variation; DXA, dual energy X-ray absorptiometry; ELISA, enzyme linked immune-sorbent assay; HCl, hydrochloric acid; nCi, nanocurrie; NH_4OH, ammonium hydroxide; NDSR, Nutrition Data System for Research; qCT, quantitative computed tomography; RCT, randomized controlled trial; RDA, recommended dietary allowances; PTH, parathyroid hormone; WHNRC, Western Human Nutrition Research Center.

Abstract (Word count = 280)
Background: Few interventions directly compare equivalent calcium and vitamin D from dairy vs. supplements on the same bone outcomes. The radioisotope calcium-41 (^{41}Ca) holds promise as a tracer method to directly measure changes in bone resorption with differing dietary interventions.

Objective: Using ^{41}Ca tracer methodology, determine if 4 servings/d of dairy foods results in greater ^{41}Ca retention than an equivalent amount of calcium and vitamin D from supplements. Secondary objective was to evaluate the time course for the change in ^{41}Ca retention.

Methods: In this crossover trial, postmenopausal women (n=12) were dosed orally with 100nCi of ^{41}Ca and after a 180 day equilibration period received dairy (4 servings/d of milk or yogurt; ~1300 mg calcium, 400 IU cholecalciferol (vitamin D_3/d)) or supplement treatments (1200 mg calcium carbonate/d and 400 IU vitamin D_3/d) in random order. Treatments lasted 6 wks separated by a 6 wk washout (WO). Calcium was extracted from weekly 24 hr urine collections; accelerator mass spectrometry (AMS) was used to determine the $^{41/40}$Ca ratio. Primary outcome was change in $^{41/40}$Ca excretion. Secondary outcome was the time course for change in ^{41}Ca excretion during intervention and WO periods.

Results: The $^{41/40}$Ca ratio decreased significantly over time during both treatments; there was no difference between treatments. Both treatments demonstrated a significant retention of ^{41}Ca within 1-2 wks ($p = 0.0007$ and $p < 0.001$ for dairy and supplements, respectively). WO demonstrated a significant decrease ($p = 0.0024$) in ^{41}Ca retention within 1-2 wk, back to pre-intervention levels.

Conclusion: These data demonstrate that urinary ^{41}Ca retention is increased with an increase in calcium and vitamin D intake regardless of the source of calcium, and the increased retention occurs within 1-2 wks.

Key words: ^{41}Ca, dairy, calcium supplement, kinetic model, postmenopausal.

Introduction

Currently an estimated 10.2 million adults age 50 and older in the United States have osteoporosis, while an estimated 43.5 million have low bone mass [1]. Health care costs related to osteoporotic fractures are projected to reach $25.3 billion in the U.S. by 2025 [2]. Calcium and vitamin D are widely considered the most critical nutrients for osteoporosis prevention and treatment [3]. Calcium absorption in the small intestine declines with age, and women lose approximately 200 mg of calcium per day in the first 3-4 years of menopause, followed by approximately 45 mg lost per day in the next 5-10 years [4]. Calcium can be lost in the feces, urine, skin, hair, nails, and digestive secretions [5, 6].

Many population groups in the West fall short of the recommended dietary calcium intake, particularly postmenopausal women [7]. The Institute of Medicine now recommends 1200 mg of calcium per day for women 51 years and older [8], but average intakes for American women are estimated to be 750- 850 mg of calcium per day [5]. Calcium supplement use is common: nearly 60% of women in the US take a calcium supplement [9]. Evidence to support the use of calcium and/or vitamin D supplements for fracture prevention is the subject of debate [9, 10]. In light of the numerous concerns related to calcium supplements, nutrition experts frequently encourage patients to consume calcium via food, especially dairy products [9]. The 2015-2020 Dietary Guidelines for Americans recommend three cups of low fat or fat free milk (or equivalent dairy products) per day for bone health in persons nine years old and above [11]. Some observational studies have shown no positive effects of dairy intake on bone in older adults [12-14]. In contrast, other observational studies support a benefit of dairy products on bone health [15, 16].

Randomized controlled trials (RCT) using dairy foods have reported a beneficial impact on markers of bone turnover [17, 18] as well as on bone mineral density (BMD) [19, 20] in postmenopausal women, but very limited work has compared equivalent amounts of calcium from dairy foods vs. supplements on the same bone outcome variables [21- 23].

Current methods of assessing bone health have limitations. Dual energy X-ray absorptiometry (DXA) is used clinically to monitor BMD, but is limited by low sensitivity as well as the inability to capture all aspects of bone strength. Image quality and spatial resolution of the scans are poor, and six months to two years are required to detect significant changes in BMD after an intervention. Quantitative computed tomography (qCT) scans of bone, which measure volumetric BMD, are more sensitive and precise than DXA, but expose subjects to a greater radiation dose [24]. Biomarkers of bone turnover can be measured to assess bone remodeling rates. Although biomarkers of bone turnover require less time than imaging techniques to see the effects of an intervention, they are limited by high inter- and intra-individual variation [25, 26].

The radioisotope ^{41}calcium (^{41}Ca), in conjunction with accelerator mass spectrometry (AMS), holds promise as a technique to measure small changes in calcium retention in a short period of time. In brief, calcium kinetic studies can be done to monitor appearance and disappearance of the tracer in a variety of biological specimens [27]. These techniques reveal changes in bone accretion and resorption in response to experimental interventions. Because bone turnover is slow the 41Ca isotope can be used *in vivo* to deep label the skeleton. The ^{41}Ca isotope has a half-life of 1.03×10^5 years, negligible radiological risk to human and can be used to assess short term changes in ^{41}Ca retention with a variety of interventions following a single small dose [26, 27]. Following an oral dose, the ^{41}Ca tracer is taken up into a short-term pool where it awaits incorporation into the skeleton. The tracer not incorporated into the skeleton is gradually

excreted from the short-term pool into urine; this takes approximately 6 months [28]. After the 6 month equilibration period any tracer in the body resides in mineralized bone, and the appearance of ^{41}Ca in urine is a direct result of bone turnover (^{41}Ca being lost from the skeleton). The amount of ^{41}Ca lost from the skeleton is evaluated by comparing the tracer excretion curve extrapolated from equilibration data prior to intervention to the tracer excretion curve during intervention [29, 30]. In addition, subjects can also serve as their own controls in crossover studies, so smaller sample sizes can be used [29]. To date, this method has not been employed in a nutritional intervention with dairy foods.

Aims

The aims of this study were to 1) determine if 4 servings/d of dairy foods increases ^{41}Ca retention more than an equivalent amount of calcium & vitamin D from supplements. Secondary objective was to evaluate the time course for change in ^{41}Ca retention in healthy postmenopausal women.

Materials and Methods

Subjects

A total of 12 healthy women at least two years post-menopause completed the study protocol. Subjects ranged in age from 50-65 years, were weight stable (±2.3 kg in the past three months), and were classified as low dairy consumers ($\leq$ 2.5 serves/d). Exclusion criteria included the use of oral hormone therapy in the past year, BMD T scores > 0 or < -1.5, use of calcium or vitamin D supplements, autoimmune or inflammatory disorders, history of non-traumatic bone fracture, and lactose intolerance. Subjects were recruited from the community via flyers, newspaper ads, and email list serves as well as informational booths at local farmers' markets. The study was

conducted at the USDA, Western Human Nutrition Research Center (WHNRC) on the University of California, Davis campus and was approved by the Institutional Review Boards of the University of California, Davis (#22919-7) and Lawrence Livermore National Laboratory (LLNL) (#11-008). A CONSORT diagram of enrollment and follow up of study subjects is shown in Figure 1. All study participants gave informed consent prior to starting the study protocol. The study was registered at clinicaltrials.gov as NCT01394484.

Study Design

The study design is summarized in Figure 2. After enrollment, subjects received an oral labeling dose of 100nCi ^{41}Ca and began a 180 day equilibration period during which the isotope excretion stabilized to a steady rate of natural loss. Subjects were instructed to continue their normal low dairy diet and lifestyle during this time. Subjects provided 24 hr urine collections prior to the ^{41}Ca dose (day 0), and during the equilibration period at days 90, 120, 150, and 180.

Following the 180 day equilibration period subjects were randomized to one of two 42 day interventions followed by a 42 day washout (WO) period. After completion of the initial intervention and WO period women continued the study on the second intervention. The dairy intervention included 20 (1 cup/237 mL) servings of milk (1% fat, 400 mg calcium and 100 International Units (IU) cholecalciferol (vitamin D_3)/ serving) per week and 8 (8 oz/227 g) servings of yogurt (low fat vanilla, 200 mg calcium and 100 IU vitamin D_3 per serving) per week. Women were instructed to consume the dairy foods throughout the day. Supplement intervention included a calcium supplement tablet (600 mg calcium per tablet; Caltrate) twice daily and a vitamin D supplement tablet (400 IU vitamin D_3 per tablet) once daily. No other supplements were permitted.

Dairy provided ~1300 mg calcium and 400 IU vitamin D_3/ d, and Supplements provided 1200mg calcium and 400 IU vitamin D_3/d. Subjects were instructed by the study dietitian how to adjust their food intake to account for the energy associated with the dairy servings (~350 kcal per day) and where dairy foods could be included in their diets, e.g. coffee latte for 1 serving of milk. Subjects were instructed to follow their usual (low dairy diet) during the supplement and WO periods.

Subjects completed 24 hr urine collections for the measurement of the excreted ^{41}Ca, as well as other urinary minerals. Urine collections were made at the beginning and at weekly intervals for each intervention and WO phase; for a total of 24 (24 hr) urine collections. Blood was drawn at ~0800, following an overnight fast of 10 hr., at the beginning and end of each intervention period.

Compliance

During the intervention periods, subjects reported to the WHNRC weekly to pick up the dairy products or supplement tablets. Compliance to the interventions was measured via empty milk and yogurt containers that were returned and by pill count on returned supplement foil-blister packs. Additionally, diet records were kept and reviewed for compliance by the study dietitian weekly when women returned to the WHNRC to pick dairy or supplement supplies.

Dietary Assessment

Women kept 3-day food logs each week for each intervention and the WO period. The first day of the first diet record was randomly assigned, and thereafter the days of the week progressed sequentially. For example, day 1 diet record might start on a Wednesday and continue through Friday of week 1. The diet record for the second week of the intervention would then start on

Saturday and continue through Monday. This "rolling" sequence was continued throughout the entire protocol. This provided an equal number of records for each day of the week for the course of the study; a total of 8 days for each day of the week: 8 Sundays, 8 Mondays, 8 Tuesdays, etc. for a total of 56 days. During the dairy intervention, women also recorded the number of servings they consumed of the milk and yogurt provided each day, as well as other dairy foods consumed. During the supplement intervention women recorded their daily supplement consumption in their diet log. In addition, women were interviewed by a registered dietitian to verify their food records.

The Nutrition Data System for Research (NDSR, University of Minnesota, 2011) was used to analyze the diet records for energy, macronutrients, calcium, phosphorus, magnesium, sodium, potassium, saturated fat, monounsaturated fat, polyunsaturated fat, trans fat, vitamin D, and servings of dairy, fruit, vegetables, grains, meat, nuts and seeds.

Anthropometric Measurements

Anthropometric measurements were taken for each woman by a trained research assistant. Body weight was measured to the nearest 0.1 kg using an electronic scale (Circuits and Systems Inc, E. Rockaway, NY). Standing height, without shoes, was measured to the nearest 0.1 cm with a wall-mounted stadiometer (Ayrton stadiometer, model S100; Ayrton Corp, Prior Lake, MN). Body mass index (BMI) was calculated based on weight and standing height measurements and expressed as kg/m^2.

Body Composition and Bone Mineral Density

Body composition (lean mass and fat mass of the total body) as well as bone mineral content (BMC), and areal BMD of the lumbar spine and hip were measured with a Delphi-W QDR DXA

bone densitometer (Hologic Inc, Bedford, MA). Calibration procedures were carried out daily according to manufacturer instructions. The coefficient of variation (CV) for the DXA instrument during the course of the study was 0.457% for the lumbar spine BMD calibration phantom. All DXA scans were analyzed by a single operator to decrease the variance in the measurement data.

Accelerator Mass Spectrometry: Measurement of ^{41}Ca

Calcium was extracted from each of the 24h urines collected and used to determine the $^{41/40}Ca$ ratio. The calcium extraction for the ^{41}Ca tracer has been previously described [27]. Briefly, samples were converted to acid solution with HCl to pH <1.9. Acid soluble calcium from the 14 urine samples was converted to calcium oxalate by adding saturated ammonium oxalate solution. Concentrated ammonium hydroxide (NH_4OH) was used to adjust the samples to pH 10 and thus promote the release of less soluble metal oxalates. The oxalate pellet was re-suspended in acid, and calcium was isolated from other cations using cation exchange chromatography. Concentrated hydrofluoric acid (28.9 molarity) was added to yield calcium fluoride, which was then pelletized by centrifugation and washed with de-ionized water. Samples were dried at 100 degrees Celsius for 20 hr in a muffle furnace. Samples were shipped from the WHNRC to the LLNL (Livermore, CA). A small amount of niobium powder (1 part Nb:4 parts CaF_2 by mass) was added to increase thermal and electrical conductivity for ion beam stability in the AMS source. Primary isotopic standards, secondary standards, and backgrounds were prepared at LLNL following previously described methods [27]. Acidic solutions with known $^{41}Ca/^{40}Ca$ ratios were used to precipitate calcium fluoride by the addition of concentrated hydrofluoric acid, followed by centrifugation, rinsing with de-ionized water, and drying overnight in a muffle furnace at 100c. The use of the ^{40}Ca in the $^{41}Ca/^{40}Ca$ standards is important because it represents

urinary calcium excretion from all sources, e.g. dietary intake, compared to the ^{41}Ca whose only source, after equilibration, is from the skeleton. All samples and standards were analyzed on the HVEE-FN-class AMS system at LLNL operated as described [30]. Greater than 88% of the normalized primary standards were within 5% of the published value [31]. Average repeatability of the secondary standards was generally 1-7% for $^{41}Ca/^{40}Ca$ ranging $9x10^{-9}$ to $9x10^{-12}$.

Statistical Analysis

Sample size calculation for the 12 subject study was based on the assumption that the change in the $^{41/40}Ca$ ratio is not correlated within-subjects and that the standard deviation of the change in the ratio is 10 percentage points and resulted in an 80% power to detect a 12.6% change in urinary $^{41/40}Ca$ over time.

The change in the $^{41/40}Ca$ ratio was the primary outcome variable for this study. Evaluation of the calcium loss was based on the $^{41/40}Ca$ urinary excretion from the weekly 24-hr urine collections over the 6 wks of each intervention and the WO. Therefore, each period (intervention and WO) had 6 urinary data points, one per week for the duration of the 6-wk intervention. Data were coded for order effect (dairy first or supplements first) and analyzed by analysis of variance (ANOVA) to determine if an order effect existed. ANOVA was also used to determine if significant differences existed between dairy and supplement interventions in the urinary $^{41/40}Ca$ excretion over the 6-wk intervention (treatment by time interaction).

Continuous variables were assessed for normality using Shapiro-Wilk and D'Agostino-Pearson normality tests and were transformed as appropriate. Nonparametric tests were used on data that were not conducive to transformation. Descriptive statistics were performed on pre-^{41}Ca dose

baseline characteristics. The secondary outcome of time to change in $^{41/40}$Ca was evaluated by ANOVA using the difference between weekly $^{41/40}$Ca values.

Additionally, to test whether the $^{41/40}$Ca excretion ratio was different from what would be expected in the case of no intervention the kinetic model developed by Denk et al. [28] was used to establish the predicted curve of ^{41}Ca loss from the ^{41}Ca excretion data during the 180 equilibration period plus the values during the WO. The actual values during the two interventions were compared to the predicted values with a mixed model analysis that included type of measurement (actual vs. predicted), intervention (dairy vs. supplement), week of intervention (1 through 6), and period of intervention (first vs. second) and all of their estimable interactions as main effects, and subject as a random effect. SAS for Windows Release 9.4 (SAS Institute, Inc, Cary, NC) and GraphPad Prism 6.0c (GraphPad Software, Inc, La Jolla, CA) were used for statistical analyses.

Results

Subject characteristics at baseline (prior to the ^{41}Ca dose on day 0) are shown in Table 1. All subjects were healthy postmenopausal women with hip and spine bone densities at the low end of the normal range. Compliance to treatment regimens was 100% for both phases.

The ratio of urinary $^{41/40}$Ca excretion over time for each treatment is shown in Figure 3. There was a significant decline in the $^{41/40}$Ca excretion during Intervention I compared to the predicted value with no intervention. Conversely, there was a significant increase in the $^{41/40}$Ca excretion during WO returning to pre-intervention or untreated levels. During Intervention II the $^{41/40}$Ca excretion declined significantly a second time. The response to either Intervention or WO was observed within 1-2 wks and continued to decline throughout the 6 weeks with the maximum

reduction occurring by week 6 (Table 2; Figure 3). The urinary ^{41}Ca excretion decreased during both interventions, confirming a fast-exchangeable pool suggested in kinetic models [29, 32]. During WO the excretion increased also within 1 week of withdrawal of calcium and vitamin D intakes representing a return to "normal" pre-intervention levels of excretion (Table 2). Repeated measures ANOVA revealed a significant time effect during both intervention periods and the WO in the $^{41/40}$Ca ratio response to treatment or WO (Table 2).

Typical with increased calcium intake is increased calcium excretion, this study was no different. Dietary calcium increased ~170% and urinary calcium was elevated during the intervention periods (179 ± 59 mg vs. 150 ± 60 mg) compared to the value just before the intervention (Figure 4). This translates to a 20.7% increase in urinary calcium during interventions and only a small portion of the increased calcium intake. However, the change in ^{41}Ca was not entirely explained by the increased total urinary excretion and a possible dilution effect. Figure 4 also shows that during weeks 3, 4, 5, and 6 urinary Ca trends back toward pre-intervention levels from a high of about 21% at week 2 to just 6% at week 6. This may be interpreted as the body adjusting to the increased Ca load. $^{41/40}$Ca during weeks 3-6 show a much more stable trend dropping from -16% at week 2 to -19% at week 6; a 3% higher ^{41}Ca retention in bone pools by the end of the first intervention. Results for the second intervention were not different from those observed in the first intervention. The 3% observed retention in ^{41}Ca could be due to either decreased resorption or increased formation. Given that both dairy and supplement interventions demonstrated no change in BAP (bone alkaline phosphatase), but did show a decrease in CTx (carboxy-terminal collagen crosslinks) (Supplemental Table 2), we suggest that the observed ^{41}Ca retention was most likely due to decreased resorption.

Discussion

By measuring the change in $^{41/40}$Ca in urine by AMS we have demonstrated that interventions using calcium and vitamin D from either dairy foods or supplements exert equal effects on calcium metabolism. Furthermore, our study is the first to show that the rapid turn-over pool suggested in calcium kinetic model literature [32] is responsive to a calcium and vitamin D intervention, whether from food or supplements, as quickly as one week.

The ^{41}Ca - AMS method has been used to assess the effect of bisphosphonates in postmenopausal women [29], in a comparison of healthy vs. end stage renal disease patients [30] as well as in a comparison of different isoflavone sources in postmenopausal women [32]. The present study is the first to utilize the ^{41}Ca isotope with AMS quantification in an intervention with dairy foods vs. supplements. Denk and colleagues [29], in a study using bisphosphonates with postmenopausal women, present a 3-compartment kinetic model similar to that of Wastney et al. [32] in adolescent girls and young women. In both studies the authors indicate that compartment 2, the fast exchange pool, serves as a transfer station to deposit into or remove calcium from a slower turnover pool – presumably the skeleton. Wastney and coauthors provide kinetic details, after oral and intravenous administration of calcium isotopes, in serum, urine and feces in adolescent girls compared to young women and demonstrated higher rates of absorption, lower rates of excretion, higher turnover of bone and higher calcium retention in the girls vs. the young women. They did not conduct an intervention to determine responsiveness in the rapidly exchanging compartment 2 to calcium and vitamin D use.

Denk and colleagues [29] examined changes in calcium kinetics to bisphosphonates therapy, not dietary or supplement calcium and vitamin D, and only analyzed urinary $^{41/40}$ Ca ratio at 2, 4, 6 and 8 weeks after administration and monthly thereafter. The urine collections at these intervals did not allow for the examination of the shorter more responsive fast turn-over pool.

Schild et al. [34] also using ^{41}Ca labeling method found that urinary ^{41}Ca retention was increased with increasing levels of daily vitamin D supplementation taken daily for 3 months by healthy postmenopausal women. Supplementation was positively associated with a downward shift in the urinary $^{41/40}$ Ca ratio compared to the predicted change without intervention. Schild et al. also demonstrated that increasing levels of vitamin D affected the transfer rate from the central compartment to a fast exchange compartment. They hypothesized that the fast exchange compartment represented an exchange from the extracellular space to the surface of the bone. Our results also demonstrate the rapidity with which the fast exchangeable pool responds to increases or decreases in calcium and vitamin D intake.

Our findings of no difference in calcium retention between food vs. supplemental intake of calcium and vitamin D is in contrast with those of Recker and Heaney [21] who reported that both a low-fat milk and calcium carbonate supplement improved calcium balance in 30 healthy postmenopausal women, but calcium carbonate suppressed bone remodeling to a greater extent than milk. However, this conclusion was based on data from two separate studies and was, therefore, not a direct comparison.

More consistent with the present findings were those of Prince and colleagues [22], who conducted a two-year study of 168 postmenopausal women and evaluated the effects three different calcium treatments or placebo on BMD. Calcium lactate gluconate tablets and skim milk powder significantly attenuated bone loss at certain sites (inter-trochanteric hip, ultradistal tibia) compared to placebo, but differences between these treatments were not significant. The investigators concluded that the milk powder and calcium carbonate were essentially equivalent in preventing bone loss [22]. Later, Manios et al. [23] reported a 12 month RCT of 101

postmenopausal Greek women and compared three servings of low fat dairy to calcium and vitamin D supplementation and a control (usual) diet. Of the three groups, dairy consumption led to the greatest attenuation of bone resorption (a 23% decrease in CTx). Unlike our study, the dairy group had significantly greater BMD at the pelvis, total spine and total body after 12 months compared to the supplement and control groups, suggesting an advantage of dairy treatment on multiple parameters of bone health [23].

Neither the present study nor the study by Prince and colleagues [22] showed robust differences in the anti-resorptive effects of the calcium supplement versus dairy foods, but advantages of dairy become evident when dietary intake data are examined. In the present study, subjects consumed significantly greater amounts of protein, carbohydrate, vitamin A, zinc and potassium during the dairy treatment than during either the supplement intervention or WO (Supplemental Table 1). The dairy intervention also led to significant increases in dietary intake of folate, phosphorus, and magnesium compared to the subjects' typical intake (WO). These nutrients are well recognized as bone-enhancing nutrients [3, 7, 33] and, over a longer duration, may result in a shift toward a significant difference between food and supplement treatments.

It is possible that the six week time frame of the present study was too brief to observe mineralization changes represented by bone formation markers such a bone alkaline phosphatase (BAP) (Supplemental Table 2). The lack of change in BAP is consistent with a study of postmenopausal women by Bonjour and colleagues [18]. This 16-week crossover trial compared treatment with 1200mg/day calcium from semi skimmed milk vs. no milk supplement and found significant changes in bone formation markers amino-terminal propeptide of type 1 procollagen (P1NP) and osteocalcin, but not in BAP. Likewise, no significant PTH change during either treatment is consistent with previous reports that calcium intake prevents increases in PTH over

time [19, 23]. Calcium tablets have been shown to significantly decrease PTH after 6 months [22], but the six week treatment duration in the present study may have been insufficient to observe this suppressive effect. The sample size of the present study is comparable to other ^{41}Ca-AMS studies by Weaver and colleagues [33] (n=11) and Denk and colleagues [29] (n=6). The crossover design allowed each woman to serve as her own control, thereby limiting inter-individual variability and allowing for a smaller sample size.

A limitation of the present study is the racial homogeneity in this sample of Caucasian women. Racial differences in bone density and bone structure are well known [6], so results of the present study cannot be extrapolated to other women of other races. An added limitation of the present study is that the vitamin D_3 dose of 400 IU/d given during both treatments does not reflect the most current RDA value of 600 IU per day for women 51-70 years old. The RDA changed from 400 IU to 600 IU while the present study was underway [8], so we proceeded with the original study protocol. Future studies using daily doses of vitamin D_3 greater than 400 IU are needed [9].

The present study is the first to use the ^{41}Ca-AMS method in a dietary intervention study with dairy products and directly compares calcium and vitamin D intake from dairy foods vs. supplements on the same bone variables. The highly sensitive ^{41}Ca-AMS technique represents an alternative method to other methods like bone turnover biomarkers that require large sample sizes and months of intervention to detect treatment differences. Both dairy products and calcium vitamin D supplements demonstrated comparable short term effect on calcium retention, but the dairy treatment provided a more nutrient dense diet for this group of postmenopausal women.

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References

1. Wright NC, Looker AC, Saag KG, Curtis JR, Delzell ES, Randall S, et al. The recent prevalence of osteoporosis and low bone mass in the United States based on bone mineral density at the femoral neck or lumbar spine. J Bone Miner Res. 2014;29(11):2520-6.
2. Cosman F, de Beur SJ, LeBoff MS, Lewiecki EM, Tanner B, Randall S, et al. Clinician's guide to prevention and treatment of osteoporosis. Osteoporos Int. 2014;25(10):2359-81.
3. Caroli A, Poli A, Ricotta D, Banfi G, Cocchi D. Invited review: Dairy intake and bone health: a viewpoint from the state of the art. J Dairy Sci. 2011;94(11):5249-62.
4. Tella SH, Gallagher JC. Prevention and treatment of postmenopausal osteoporosis. J Steroid Biochem Mol Bio. 2014;142:155-70.
5. Bauer DC. Clinical practice. Calcium supplements and fracture prevention. N Engl J Med. 2013;369(16):1537-43.
6. Heaney RP. Calcium, dairy products and osteoporosis. J Am Coll Nutr. 2000;19(2 Suppl):83s-99s.
7. Peters BS, Martini LA. Nutritional aspects of the prevention and treatment of osteoporosis. Arq Bras Endocrinol Metabol. 2010;54(2):179-85.
8. Institute of Medicine (US) Committee to review dietary reference intakes for vitamin D and calcium (2011). In: Ross AC, Taylor CL, Yaktine AL, Del Valle HB et al (eds). Dietary reference intakes for calcium and vitamin D. National Academies Press (US), Washington (DC). Available from http://www.ncbi.nlm.nih.gov/books/NBK56070/. Accessed December 2015.
9. Moyer VA. Vitamin D and calcium supplementation to prevent fractures in adults: U.S. Preventive Services Task Force recommendation statement. Ann Intern Med. 2013;158(9):691-6
10. Jackson RD, Mysiw WJ. Insights into the epidemiology of postmenopausal osteoporosis: the Women's Health Initiative. Semin Reprod Med. 2014;32(6):454-62.
11. US Department of Health and Human Services; US Department of Agriculture. *2015-2020 Dietary Guidelines for Americans*. 8th ed. Washington, DC: US Dept of Health and Human Services; December 2015. http://www.health.gov/DietaryGuidelines. Accessed January 12, 2016.
12. Feskanich D, Willett WC, Colditz GA. Calcium, vitamin D, milk consumption, and hip fractures: a prospective study among postmenopausal women. Am J Clin Nutr. 2003;77(2):504-11.
13. Wadolowska L, Sobas K, Szczepanska JW, Slowinska MA, Czlapka-Matyasik M, Niedzwiedzka E. Dairy products, dietary calcium and bone health: possibility of prevention of osteoporosis in women: the Polish experience. Nutrients. 2013;5(7):2684-707.
14. Michaelsson K, Wolk A, Langenskiold S, Basu S, Warensjo Lemming E, Melhus H, et al. Milk intake and risk of mortality and fractures in women and men: cohort studies. BMJ (Clinical research ed). 2014;349:g6015.
15. Key TJ, Appleby PN, Spencer EA, Roddam AW, Neale RE, Allen NE. Calcium, diet and fracture risk: a prospective study of 1898 incident fractures among 34 696 British women and men. Public Health Nutr. 2007;10(11):1314-20.
16. Sahni S, Mangano KM, Tucker KL, Kiel DP, Casey VA, Hannan MT. Protective association of milk intake on the risk of hip fracture: results from the Framingham Original Cohort. J Bone Miner Res. 2014;29(8):1756-62.

17. Heaney RP, Rafferty K, Dowell MS. Effect of yogurt on a urinary marker of bone resorption in postmenopausal women. J Am Diet Assoc. 2002;102(11):1672-4.
18. Bonjour JP, Brandolini-Bunlon M, Boirie Y, Morel-Laporte F, Braesco V, Bertiere MC, et al. Inhibition of bone turnover by milk intake in postmenopausal women. Br J Nutr. 2008;100(4):866-74.
19. Chee WS, Suriah AR, Chan SP, Zaitun Y, Chan YM. The effect of milk supplementation on bone mineral density in postmenopausal Chinese women in Malaysia. Osteoporos Int. 2003;14(10):828-34.
20. Moschonis G, Katsaroli I, Lyritis GP, Manios Y. The effects of a 30-month dietary intervention on bone mineral density: the Postmenopausal Health Study. Br J Nutr. 2010;104(1):100-7.
21. Recker RR, Heaney RP. The effect of milk supplements on calcium metabolism, bone metabolism and calcium balance. Am J Clin Nutr. 1985;41(2):254-63.
22. Prince R, Devine A, Dick I, Criddle A, Kerr D, Kent N, et al. The effects of calcium supplementation (milk powder or tablets) and exercise on bone density in postmenopausal women. J Bone Miner Res. 1995;10(7):1068-75.
23. Manios Y, Moschonis G, Trovas G, Lyritis GP. Changes in biochemical indexes of bone metabolism and bone mineral density after a 12-mo dietary intervention program: the Postmenopausal Health Study. Am J Clin Nutr. 2007;86(3):781-9.
24. Beck T. Measuring the structural strength of bones with dual-energy X-ray absorptiometry: principles, technical limitations, and future possibilities. Osteoporos Int. 2003;14 Suppl 5:S81-8.
25. Civitelli R, Armamento-Villareal R, Napoli N. Bone turnover markers: understanding their value in clinical trials and clinical practice. Osteoporos Int. 2009;20(6):843-51.
26. Civitelli R, Armamento-Villareal R, Napoli N. Bone turnover markers: understanding their value in clinical trials and clinical practice. Osteoporos Int. 2009;20(6):843-51.
27. Lin Y, Hillegonds DJ, Gertz ER, Van Loan MD, Vogel JS. Protocol for assessing bone health in humans by tracing long-lived 41Ca isotope in urine, serum, and saliva samples. Anal Biochem. 2004;332(1):193-5.
28. Denk E, Hillegonds D, Vogel J, Synal A, Geppert C, Wendt K, fattinger K, Hennessy, Berglund M, Hurrell RF, Walczyk. Labeling the human skeleton with ^{41}Ca to assess changes in bone calcium metabolism. Anal Bioanl Chem 2006; 386: 1587-1602.
29. Denk E, Hillegonds D, Hurrell RF, Vogel J, Fattinger K, Hauselmann HJ, et al. Evaluation of 41calcium as a new approach to assess changes in bone metabolism: effect of a bisphosphonate intervention in postmenopausal women with low bone mass. J Bone Miner Res. 2007;22(10):1518-25.
30. Fitzgerald RL, Hillegonds DJ, Burton DW, Griffin TL, Mullaney S, Vogel JS, et al. 41Ca and accelerator mass spectrometry to monitor calcium metabolism in end stage renal disease patients. Clin Chem. 2005;51(11):2095-102.
31. Nishiizumi K, Caffee MW, DePaolo DJ. Preparation of Ca-41 AMS standards. Nucl. Instrum Methods Phys Res B. 2000;172:399-403.
32. Wastney ME, Ng j, Smith d, Martin BR, Peacock M, Weaver CM. Differences in calcium kinetics between adolescent girls and young women. Am J Physiol. 1996; R208-R216.
33. Weaver CM. Should dairy be recommended as part of a healthy vegetarian diet? Point. Am J Clin Nutr. 2009;89(5):1634s-7s.

34. Schild A, Herter-Aeberli I, fattinger K, Anderegg S, Schulz-König , Vockenhuber C, et al. Oral vitamin D supplements increase serum 25-hydroxyvitamin D in postmenopausal women and reduce bone calcium flux measured by ^{41}Ca skeletal labeling. J Nutr. 2015; 145:2333-40. doi: 10.3945/jn.115.215004.

Figure Legend.

Figure 1. Enrollment and follow up of participants in the randomized crossover trial.

Figure 2. Study design. After enrollment, women received a minute labeling dose of ^{41}calcium, which was incorporated into the skeleton over a period of 180 days. Women were then randomly assigned to either the dairy or supplement interventions for 42 days. After a 42 day WO period, subjects completed the second intervention for 42 days.

Figure 3. $^{41/40}$Ca excretion ratio over time (n=12) for each intervention period and WO. Significant differences were observed by week 1 of each intervention periods I, II and wash out (0.0007, 0.0056, < 0.0001, respectively)

Figure 4. Weekly least-square means for urinary ^{40}Ca, ^{41}Ca and the $^{41/40}$Ca ratio during interventions.

Figure 1

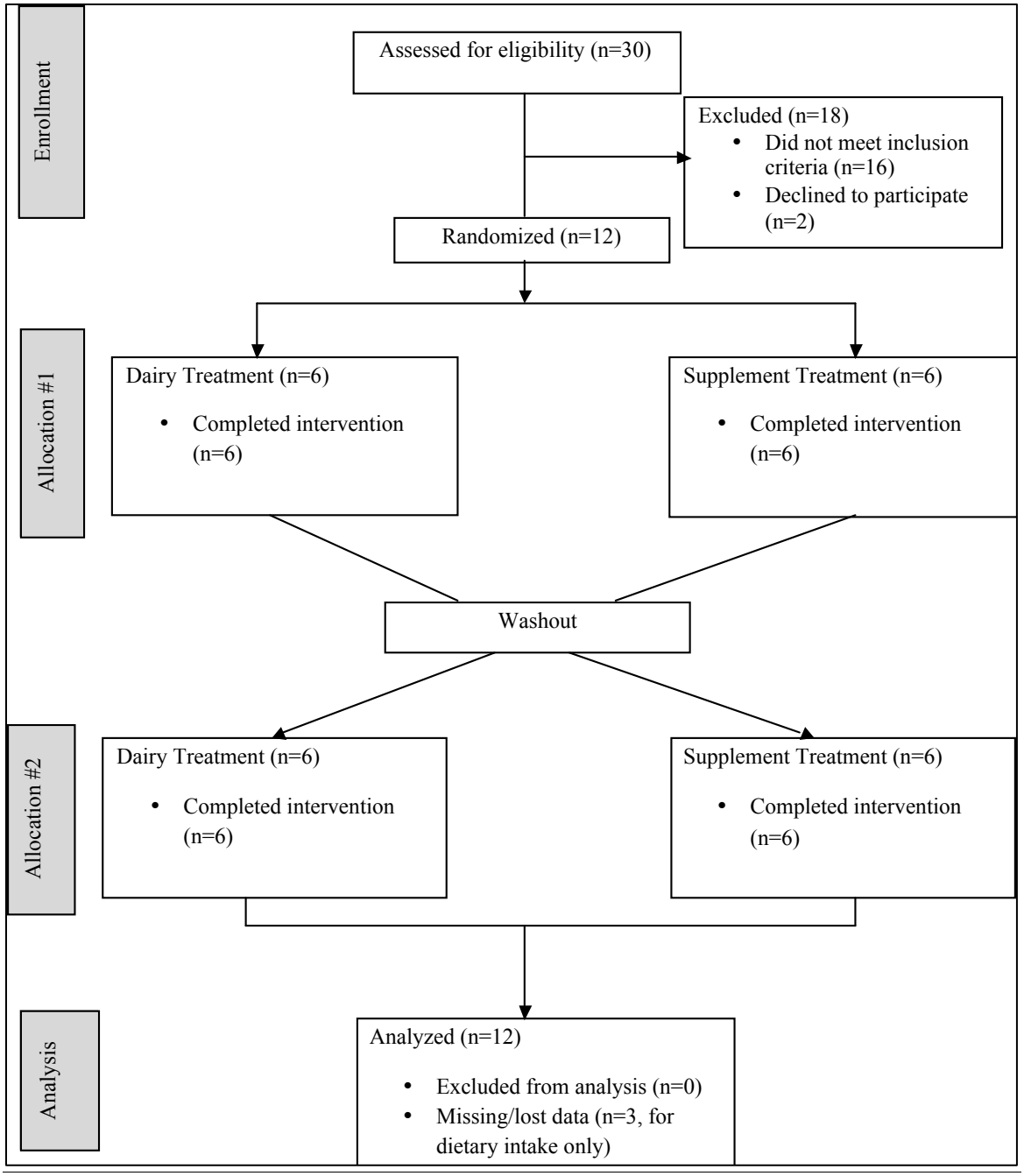
Enrollment
Assessed for eligibility (n=30)
Excluded (n=18)
• Did not meet inclusion criteria (n=16)
• Declined to participate (n=2)
Randomized (n=12)
Allocation #1
Dairy Treatment (n=6)
• Completed intervention (n=6)
Supplement Treatment (n=6)
• Completed intervention (n=6)
Washout
Allocation #2
Dairy Treatment (n=6)
• Completed intervention (n=6)
Supplement Treatment (n=6)
• Completed intervention (n=6)
Analysis
Analyzed (n=12)
• Excluded from analysis (n=0)
• Missing/lost data (n=3, for dietary intake only)

Figure 2

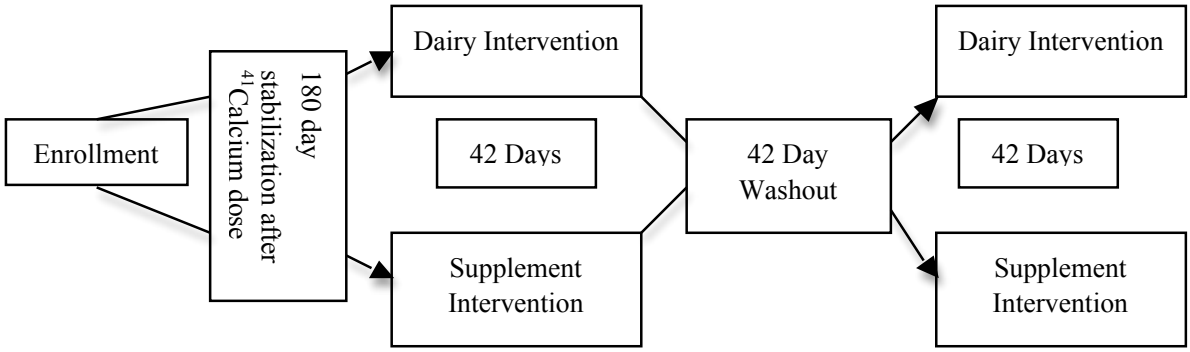
Enrollment
180 day stabilization after ^{41}Calcium dose
Dairy Intervention
42 Days
Supplement Intervention
42 Day Washout
Dairy Intervention
42 Days
Supplement Intervention

Figure 3

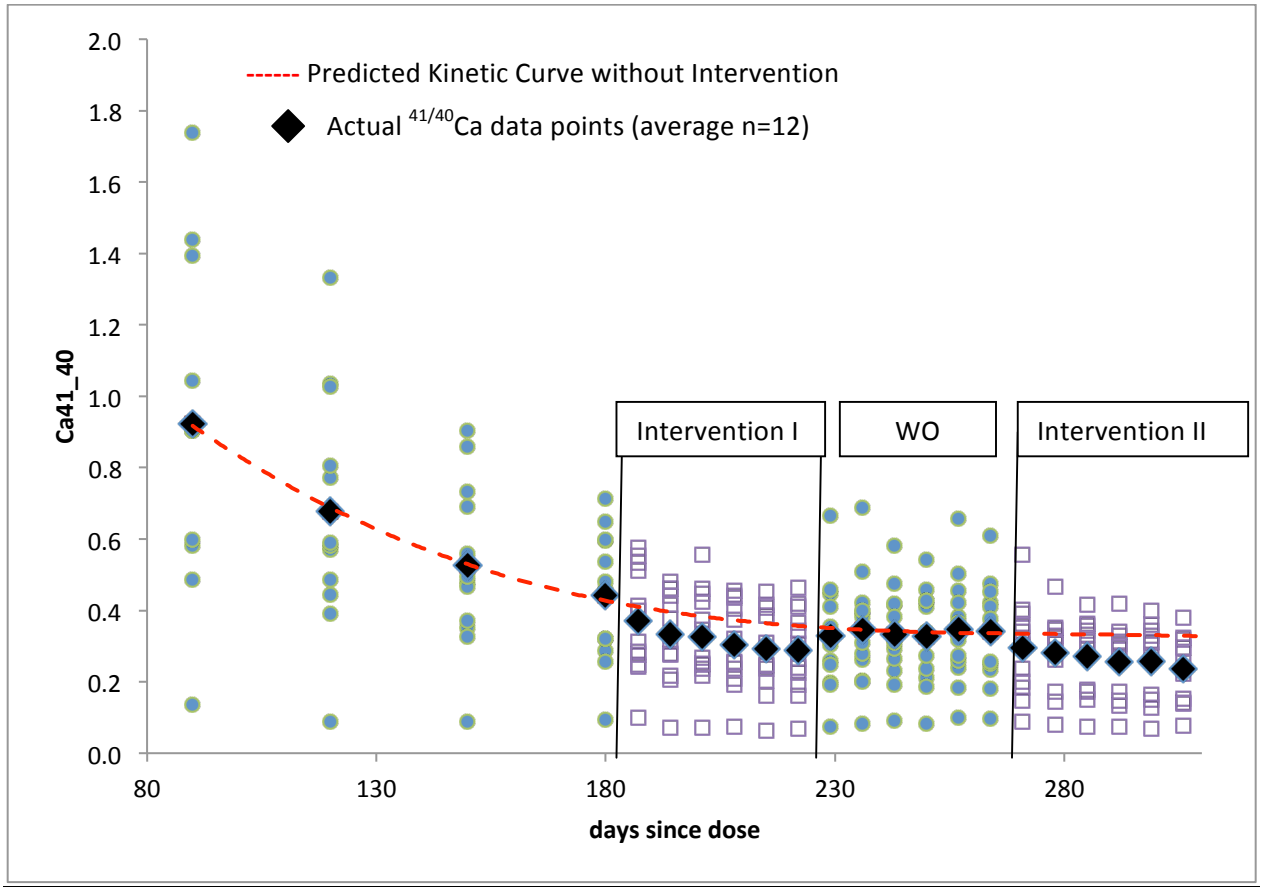
Predicted Kinetic Curve without Intervention
Actual 41/40Ca data points (average n=12)
Ca41_40
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
80
130
180
230
280
days since dose
Intervention I
WO
Intervention II

Figure 4

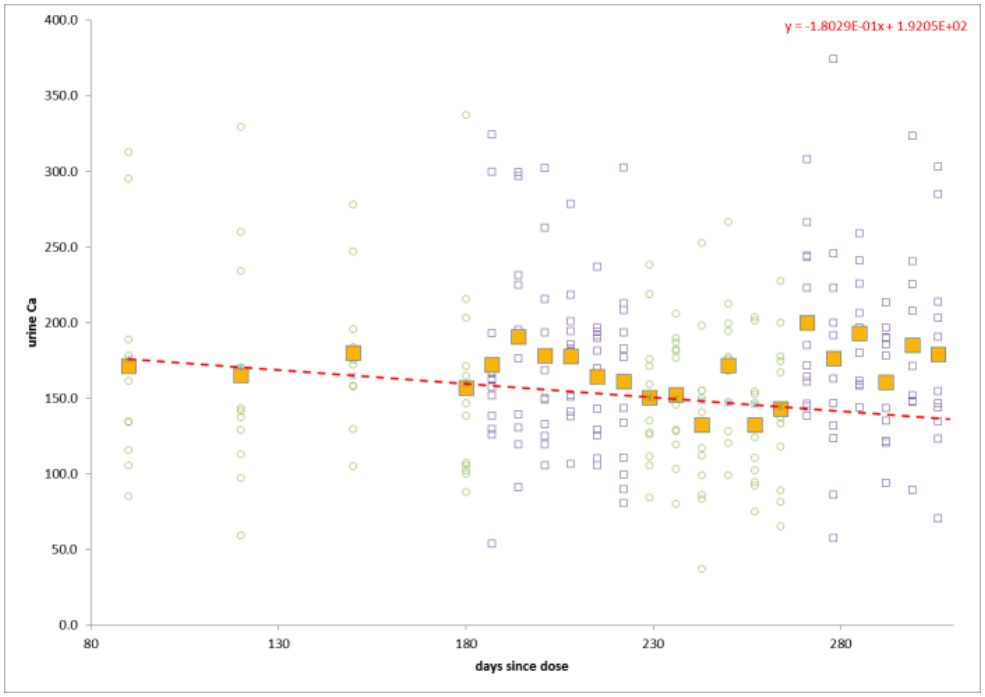
y = -1.8029E-01x + 1.9205E+02
400.0
350.0
300.0
250.0
200.0
150.0
100.0
50.0
0.0
urine Ca
80
130
180
230
280
days since dose

Table 1. Subject characteristics at baseline (n=12 females)

Parameter	Mean ± Standard Deviation
Age (years)	55.4 ± 2.5
Height (cm)	163.0 ± 4.9
Weight (kg)	63.9 ± 8.5
BMI (kg/m^2)	24.2 ± 3.7
Body Fat (%)	38.0 ± 7.4
Fat-Free Mass (kg)	37.2 ± 3.2
Spine BMC (g)	45.6 ± 4.1
Spine BMD (g/cm^2)	1.1 ± 0.1
Spine T-Score	-0.82 ± 0.62
Hip BMC (g)*	27.0 ± 2.4
Hip BMD (g/cm^2)*	0.9 ± 0.1
Hip T-Score*	-0.88 ±0.06
Systolic Blood Pressure (mm Hg)	117 ± 21
Diastolic Blood Pressure (mm Hg)	75 ± 14
PTH (pmol/L)	59 ± 19

*Average of right and left hip values

Table 2. Change in $^{41/40}$ Ca Excretion During Intervention and Wash Out Periods

Intervention - I

Days – Week	N	Mean	Standard Deviation	Probability
187 – week 1	12	-0.157	0.166	0.0007
194 – week 2	12	-0.271	0.0922	< 0.0001
201 – week 3	12	-0.299	0.136	< 0.0001
208 – week 4	12	-0.356	0.117	< 0.0001
215 – week 5	12	-0.411	0.137	< 0.0001
222 – week 6	12	-0.418	0.132	< 0.0001

Wash Out

Days – Week	N	Mean	Standard Deviation	Probability
229 – week 1	12	0.116	0.103	0.0024
236 – week 2	12	0.168	0.126	0.0007
243 – week 3	12	0.153	0.112	0.0006
250 – week 4	12	0.134	0.0931	0.0004
257 – week 5	12	0.187	0.133	0.0005
264 – week 6	12	0.176	0.122	0.0004

Intervention – II

Days – Week	N	Mean	Standard Deviation	Probability
271 – week 1	12	-0.155	0.072	< 0.0001
278 – week 2	12	-0.210	0.123	0.0002
285 – week 3	12	-0.250	0.0984	< 0.0001
292 – week 4	12	-0.315	0.0929	< 0.0001
299 – week 5	12	-0.315	0.124	< 0.0001
306 – week 6	12	-0.326	0.133	< 0.0001

Supplemental Table 1. Average Nutrient Intake per Day by Treatment

	Supplement (n=9)	Washout (n=9)	Dairy (n=9)
Energy (kcal)	1746 ± 422 [a]	1593 ± 342 [a]	1967 ± 211 [b]
Total Fat (g)	69 ± 22	59 ±17	59 ± 12
Total CHO (g)	209 ± 55 [a]	199 ± 57 [a]	266 ± 39 [b] **
Total Protein (g)	73 ± 17 [a]	67 ± 2 [a]	94 ±13 [b] *
Calcium (mg)	1951 ± 222 [b]	702 ± 265 [a]	1820 ± 86 [b]
Iron (mg)	14 ± 5	13 ± 4	14 ± 4
Sodium (mg)	2635 ± 730	2391 ±575	2541 ± 282
Folate (mcg)	379 ±100 [a]	353 ± 88 [a]	451 ± 107 [b]
Vitamin A (Retinol Activity Equivalents, cg)	819 ± 161 [a]	730 ± 240 [a]	1472 ± 228 [b] ****
Saturated Fat (g)	22 ± 9	20 ± 7	21 ± 5
Cholesterol (mg)	224 ± 87	218 ± 78	240 ± 79
Vitamin D_3 (IU)	572 ± 87 [b]	184 ± 95 [a]	569 ± 65 [b]
Vitamin K (mcg)	126 ± 47	132 ± 59	144 ± 97
Phosphorus (mg)	1173 ± 229 [a]	1113 ± 225 [a]	1803 ± 366 [b]
Magnesium (mg)	323 ± 53 [a]	284 ± 59 [a]	381 ± 39 [b]
Zinc (mg)	9 ± 2 [a]	9 ± 2 [a]	13 ± 2 [b] **
Potassium (mg)	2766 ± 279 [a]	2616 ± 476 [a]	3911 ± 374 [b] ****

Values are Mean ± SD. Different letters denote significant differences between supplement and dairy. Probability levels of significance are indicated with asterisks. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

Supplemental Table 2. Serum Biomarkers Before and After Each Supplement and Dairy Treatments

	Supplement Week 1	Supplement Week 6	Dairy Week 1	Dairy Week 6
BAP (U/L)	36 ±11	36 ±12	38 ± 12	38± 11
CTx (ng/mL)	0.57 ±0.16	0.45 ±0.14**	0.60 ± 0.16	0.44 ± 0.12****
PTH (pmol/L)	57 ± 21	57± 23	62 ± 18	59 ± 24

(n=12) Means ± SD; Significant differences between weeks 1 and 6 of each treatment are indicated with asterisks. **p<0.01, ****p<0.0001